Vol. 112: 103–111, 2014
doi: 10.3354/dao02800

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 2

FREE ACCESS

Prevalence and quantification of pathogenic *Vibrio parahaemolyticus* during shrimp culture in Thailand

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ABSTRACT: *Vibrio parahaemolyticus* is a major cause of seafood-borne gastroenteritis. The human pathogenic strains possess *tdh* or *trh* or both genes. In Thai shrimp farming, the level of pathogenic *V. parahaemolyticus* contamination has not been completely characterized, although it has been identified as a risk for people who consume undercooked shrimp. In this study, the prevalence and concentration of *V. parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (*tdh*$^+$ Vp and *trh*$^+$ Vp) were investigated during shrimp culture cycles using the most probable number (MPN) method and were confirmed by PCR and the loop-mediated isothermal amplification (LAMP) techniques. The prevalence and concentration of total Vp were high in broodstock and egg samples at the start of the hatchery cycle, but the organism decreased in the subsequent larval and postlarval stages. In contrast, total Vp was low at the beginning of the pond cycle and dramatically increased during the later stages of culture. Broodstock and fresh feed were important sources of *V. parahaemolyticus*. Numbers of *tdh*$^+$ Vp and *trh*$^+$ Vp detected by the LAMP technique were much greater than those detected by the PCR technique, especially in the late stages of the pond cycle. A direct correlation between total Vp and pathogenic Vp was demonstrated only during the harvest stage. This study will be useful as a guideline to establish levels of *V. parahaemolyticus* presence which can be considered as safe during shrimp culture. In addition, it could be used to identify the source of *V. parahaemolyticus*, which has recently been reported to be one of the etiologic agents of acute hepatopancreatic necrosis disease.

KEY WORDS: *Vibrio parahaemolyticus* · *tdh* gene · *trh* gene · Shrimp culture · LAMP

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium found in marine environments worldwide. It is a leading cause of seafood-associated gastroenteritis. The most common symptoms in humans include watery diarrhea with abdominal cramps, nausea, vomiting and in some cases fever and headaches (Yeung & Boor 2004). Not all strains of *V. parahaemolyticus* are pathogenic. *V. parahaemolyticus* strains that produce the major virulence factors, a thermostable direct hemolysin (TDH) or a TDH-related hemolysin (TRH), are considered to be human pathogenic strains (Takeda 1982, Honda et. al. 1987). TDH and TRH are encoded by the *tdh* and *trh* genes, respectively. The number of *V. parahaemolyticus* isolates that possess *tdh* or *trh* genes in the environment varies, and the number determined

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depends on the location and detection techniques. However, around 90% of clinical isolates possess *tdh*, *trh*, or both genes (Deepanjali et al. 2005, Wootipoom et al. 2007, Gutierrez West et al. 2013). Most infections due to *V. parahaemolyticus* have been associated with shellfish consumption. In Hong Kong, around 40% of *V. parahaemolyticus* associated with food poisoning is caused by consumption of shrimp (Liu & Chen 2013). Investigation of 28 isolates of *V. parahaemolyticus* obtained from shrimp isolated from the south coast of Iran revealed that 5 (1.7%) and 2 (0.7%) isolates were *tdh*$^+$ and *trh*$^+$, respectively (Rahimi et al. 2010). In Malaysia, testing of 128 *V. parahaemolyticus* isolates from frozen and cultured shrimp, including some from the culture environment, revealed that 14 (10.9%) and 4 (3.1%) were positive for the *tdh* and *trh* genes, respectively, and this indicated that healthy people are at risk after consumption of raw or undercooked shrimp (Sujeewa et al. 2009).

Thailand is one of the world's leading exporters of shrimp. In shrimp farming, especially in Thailand, the level of *tdh*$^+$ or *trh*$^+$ *V. parahaemolyticus*, as well as the source of this bacterium which is present during each shrimp cultivation cycle, is still limited, although the potential for infection of consumers cannot be ruled out. The aims of this study were to investigate the prevalence and concentration of potential infectious *V. parahaemolyticus* in shrimp cultured from the hatchery to the harvest cycle and to evaluate the probability of detecting pathogenic *V. parahaemolyticus* during each of the stages of shrimp culture. In addition, we also applied the loop-mediated isothermal amplification (LAMP) technique to detect pathogenic *V. parahaemolyticus* in shrimp culture.

MATERIALS AND METHODS

Scenario study

In this study, the prevalence and concentration of *Vibrio parahaemolyticus* was evaluated by following the shrimp culturing cycle from the hatchery, to the pond, to harvest. Feed was also investigated because this factor was involved in most stages of the cultivation processes. The study was based on a multiple stage scenario from broodstock, shrimp spawn (egg), larva hatch (nauplius, protozoea and mysis), postlarva (PL) development, and juvenile (1.5 mo culture) to adult (3–4.5 mo culture) development. At each stage, the prevalence and concentration of *V. parahaemolyticus* were determined. Parameters used in this study were the presence or absence of *V. parahaemolyticus* (hereafter termed total Vp) and the presence or absence of human pathogenic *tdh*$^+$ or *trh*$^+$ *V. parahaemolyticus* (hereafter termed *tdh*$^+$ Vp and *trh*$^+$ Vp, respectively). Quantitative evaluation was expressed as a geometric mean of the most probable number (MPN) of *V. parahaemolyticus* per g or ml (MPN g^{-1} or ml^{-1}) of sample. When SD < 0.01 $\log_{10}$ MPN, it is reported as zero (0). The probability of detection of total Vp and pathogenic *V. parahaemolyticus* as well as the correlation between total Vp, *tdh*$^+$ Vp, and *trh*$^+$ Vp were also determined (see the Appendix).

Sample collection

Samples were collected from 2 different shrimp farms (*Litopenaeus vanamei* shrimp) and 2 hatcheries located in the Ranode district, Songkhla Province, Thailand, between June 2010 and February 2012. After collection, they were kept in sterile containers and brought to the laboratory as quickly as possible. The prevalence and concentration of *V. parahaemolyticus* were determined using the MPN technique for the hatchery, pond, and harvest cycles including feed. Various quantities of samples were obtained (3, 10, or 25 ml or 25 g) depending on the type of sample (Table 1). In the hatchery cycle, the broodstock (25 g), water (25 ml) and eggs (3 ml containing more than 30 000 eggs) were collected from each farm (Table 1). In addition, 3 ml (more than 25 000 larva) containing each stage of the larva (nauplii, protozoea, and mysis) was examined. For the PL stage, PL Day 1 (PL_1) and PL Day 11–15 ($PL_{11–15}$), 25 ml (containing 25–30 PL) were tested. In the pond cycle, stages of shrimp culture can be categorized as follows: PA (pond preparation), PB (water filling), PC (water treatment), PD (postlarva release), PE1 (1.5 mo culture) and PE2 (3.0 mo culture). In this cycle as well as the harvest cycle (PE3: 4.5 mo culture), water (25 ml from 5 different locations), and sediment (25 g from 3 different locations in the pond) were collected for each stage (Table 1). In addition, for PE1 to PE3, shrimp (25 g) were obtained for *V. parahaemolyticus* investigations. For the feed at least 10 ml or 25 g of natural feed, fresh feed, or formulated feed from 2 different companies were investigated.

Bacterial enumeration and confirmation

Alkaline peptone water (APW) was used as the enrichment medium for the culture of *V. parahaemo-*

Table 1. Types and quantity of sample collected at each stage of shrimp culture. PL_1: postlarva Day 1; $PL_{11–15}$: postlarva Day 11–15; PA: pond preparation stage; PB: water filling stage; PC: water treatment stage; PD: postlarva release stage; PE1: 1.5 mo culturing stage; PE2: 3 mo culturing stage; PE3: 4.5 mo culturing (harvest) stage

Cycle	Stage(s)	Sample type	No. of samples collected	Quantity of sample
Hatchery	Broodstock	Broodstock	8	25 g
		Water	20	25 ml
	Egg	Egg	4	3 ml
	Nauplii	Nauplii	4	3 ml
	Protozoea	Protozoea	4	3 ml
	Mysis	Mysis	4	3 ml
	PL_1	PL_1	4	3 ml
	$PL_{11–15}$	$PL_{11–15}$	4	3 ml
Pond	PA	Sediment	24	25 g
		Water	24	25 ml
	PB	Sediment	24	25 g
		Water	47	25 ml
	PC	Sediment	24	25 g
		Water	40	25 ml
	PD	PL	24	3 ml
		Sediment	24	25 g
		Water	40	25 ml
	PE1	Shrimp	24	25 g
		Sediment	24	25 g
		Water	40	25 ml
	PE2	Shrimp	24	25 g
		Sediment	24	25 g
		Water	40	25 ml
Harvest	PE3	Shrimp	24	25 g
		Sediment	24	25 g
		Water	40	25 ml
Feed		Natural feed[a]	28	10 ml
		Fresh feed[b]	18	25 g
		Formulated feed	32	25 g

[a]Artemia, spirulina, [b]bloodworm, sandworm, squid, shell

lyticus. Each sample was homogenized with APW, with a pH of 8.6, at a ratio of 1:10, and the supernatant was transferred for inoculation into 3 tubes to be investigated using the MPN procedure. Briefly, 10, 1, 0.1 , 0.01, or 0.001 ml of the supernatant were inoculated into APW in triplicate. Inoculation of the 0.01 and 0.001 ml samples was performed by transferring 1 ml of the 0.1 and 0.01 inocula, respectively, in 9 ml of APW. For the egg, larva, and PL stages, 1 ml of the obtained sample was transferred to the first set of MPN dilution tubes (3 tubes in each set; total amount transferred 3 ml). Inoculated tubes were incubated for 18 h at 37°C. The presence or absence of *V. parahaemolyticus* (total Vp), *tdh*$^+$ Vp, and *trh*$^+$ Vp in each tube of the enrichment culture was examined using the colony isolation-PCR method (defined as colony PCR). Briefly, 1 loopful of APW was taken from each of the tubes showing turbidity and was streaked onto CHROMagar Vibrio (CHROMagar Microbiology). After overnight incubation at 37°C, up to 5–10 mauve colonies most likely to be *V. parahaemolyticus* were selected from each CHROMagar Vibrio plate. Each of the selected colonies was inoculated into Luria-Bertani (LB) broth medium containing 1% NaCl and incubated at 37°C with shaking (160 rpm) overnight. One ml of the overnight culture was examined for the *V. parahaemolyticus toxR, tdh* and *trh* genes using the PCR technique (colony PCR) (Yamamoto et al. 2008). The presence or absence of total Vp, *tdh*$^+$ Vp, and *trh*$^+$ Vp in each tube was determined based on the detection of these 3 genes, respectively, in the selected colonies. If at least 1 colony was PCR positive, the corresponding tube and sample were considered positive for *V. parahaemolyticus* and used to calculate the MPN for the concentration of total Vp, *tdh*$^+$ Vp, and *trh*$^+$ Vp g^{-1} (or ml^{-1}) in the corresponding sample (M. Curiale, http://i2workout.com/mcuriale/mpn/index.html). However, the 5–10 mauve colonies selected by CHROMagar Vibrio using colony PCR may not represent the whole population of *V. parahaemolyticus* in the sample. Therefore, the supernatant of all turbid MPN tubes was confirmed for *tdh*$^+$ Vp and *trh*$^+$ Vp using the LAMP assay, and the numbers in the positive tube were used to calculate the MPN as described above.

LAMP assay

One ml of each positive tube was centrifuged at 900 × *g* for 1 min to remove large debris. The supernatant was collected and centrifuged at 10 000 × *g* for 5 min. This pellet was resuspended in 500 µl sterile distilled water, boiled for 10 min, then immediately cooled and centrifuged at 20 000 × *g* at 4°C for 5 min. The clear supernatant was used as the DNA template for LAMP detection of the *tdh* and *trh* genes (Yamazaki et al. 2010). Briefly, 25 µl of LAMP reaction mixture contained 1.6 µM each of the inner primers FIP and BIP, 0.2 µM each of the outer primers F3 and B3, 0.8 µM each of the loop primers LF and LB, 12.5 µl of 2 × LAMP reaction buffer, 1.0 µl of *Bst* polymerase, and 5 µl of template DNA. After being gently mixed, it was then incubated at 65°C for

60 min and inactivated at 80°C for 2 min to complete the reaction. DNA amplification was monitored with a real-time turbidimeter (RT-160C, Eiken Chemical). A reaction was considered positive when the turbidity reached 0.1 within 60 min at 650 nm for the *tdh*-LAMP and within 90 min at 650 nm for the *trh*-LAMP. The presence of a white precipitation visible to the naked eye was considered a positive result. In addition, the presence of *V. parahaemolyticus* in all positive tubes was confirmed using *tlh* as a target gene (Yamazaki et al. 2008). The sensitivity and specificity of the LAMP technique for detection of pathogenic *V. parahaemolyticus* were calculated as in a previous study (Lau et al. 2010) and compared to the PCR method. Sensitivity was defined as (number of samples positive for both techniques)/(number of samples positive for both techniques + number of samples positive for PCR but negative for LAMP), and specificity was defined as (number of samples negative for both techniques)/(number of samples negative for both techniques + number of samples positive for LAMP but negative for PCR). This approach assumes that PCR is a gold standard test.

RESULTS

Prevalence and concentration of total Vp

For the hatchery cycle, the prevalence of *Vibrio parahaemolyticus* in both the male and female broodstocks, including water in the stocking tank, was investigated in a total of 28 samples. All of them (100%) were positive for total Vp (Table 2). In addition, all the egg and nauplii samples were positive for total Vp; however, only 25–50% of the samples obtained from the protozoea, mysis, PL_1 and $PL_{11–15}$ were positive. In the pond cycle, at the PA–PD stages, 15.9–43.8% of samples were positive for total Vp, and the number of positive samples increased to 94.3% after the shrimp had been cultured for 1.5 mo (PE1) (Table 2). It was of interest that after culture for 3 mo (PE2) and 4.5 mo (PE3) (harvest cycle) all samples were positive for total Vp.

The concentration of total Vp in the broodstock, egg, larva, PL and shrimp was evaluated for each stage of the shrimp culture. The mean and standard deviation of the concentration of total Vp detected in the hatchery cycle was high in the broodstock and egg samples (1.93 ± 0.02 and 1.99 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1}, respectively) (Fig. 1), and it then decreased significantly in the larval and PL stages (−0.18 ± 0 to −0.53 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1}). However, after release of the PL into the pond at the PD stage, the number of total Vp continuously increased during the PE1 to PE3 stages (1.62 ± 0 to 2.11 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1}).

In the pond cycle, the average concentrations of total Vp in the sediment and water samples in the PA stage were 0.46 ± 0.02 and −0.21 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1}, respectively, and they then decreased in the PB to PD stages (Fig. 2). After that the total Vp continuously increased during the PE1 to PE3 stages.

Investigation of *V. parahaemolyticus* in the shrimp feed showed that 3.6 and 83.3% of total Vp were

Table 2. Prevalence of total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (*tdh*$^+$ Vp and *trh*$^+$ Vp) in shrimp culture as determined by PCR or loop-mediated isothermal amplification (LAMP), presented as 'no. of positive samples total'. PL_1: postlarva Day 1; postlarva Day 11–15; PA: pond preparation stage; PB: water filling stage; PC: water treatment stage; PD: postlarva release stage; PE1: 1.5 mo culturing stage; PE2: 3 mo culturing stage; PE3: 4.5 mo culturing (harvest) stage. MPN: most probable number; ND: not determined

Shrimp culture stage(s)	Total Vp (%)	PCR		LAMP	
		tdh$^+$ Vp (%)	*trh*$^+$ *Vp* (%)	*tdh*$^+$ *Vp* (%)	*trh*$^+$ *Vp* (%)
Hatchery					
Broodstock	28/28 (100)	5/28 (17.8)	1/28 (3.6)	5/28 (17.8)	2/28 (7.1)
Egg	4/4 (100)	0[a]/4	0/4	0/4	0/4
Nauplii	4/4 (100)	0/4	0/4	0/4	0/4
Protozoea	1/4 (25)	0/1	0/1	0/1	0/1
Mysis	1/4 (25)	0/1	0/1	0/1	0/1
PL_1	1/4 (25)	0/1	0/1	0/1	0/1
$P_{11–15}$	2/4 (50)	0/2	0/2	0/2	0/2
Pond					
PA	21/48 (43.8)	0/21	0/21	0/21	0/21
PB	12/71 (16.9)	0/12	0/12	0/12	0/12
PC	14/64 (21.9)	0/14	0/14	0/14	0/14
PD	14/88 (15.9)	0/14	0/14	0/14	0/14
PE1	83/88 (94.3)	0/83	0/83	1/83 (1.2)	0/83
PE2	88/88 (100)	0/88	0/88	10/88 (11.4)	0/88
Harvest					
PE3	88/88 (100)	0/88	0/88	14/88 (15.9)	4/88 (4.6)
Feed					
Natural	1/28 (3.6)	0/1	0/1	0/1	0/1
Fresh	15/18 (83.3)	4/15 (26.7)	0/15	8/15 (53.3)	3/15 (20)
Formulated	0/32	ND	ND	ND	ND

[a]<0.3 MPN g^{-1} or ml^{-1}

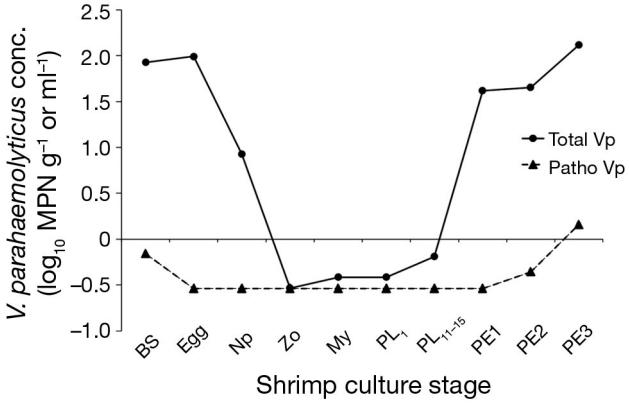

Fig. 1. Mean concentration of total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (patho Vp) in shrimp culture stages. BS: broodstock; Np: nauplii; Zo: protozoea; My: mysis; PL_1: postlarva Day 1; $PL_{11–15}$: postlarva Day 11–15; PE1: 1.5 mo culturing stage; PE2: 3 mo culturing stage; PE3: 4.5 mo culturing (harvest) stage. MPN: most probable number

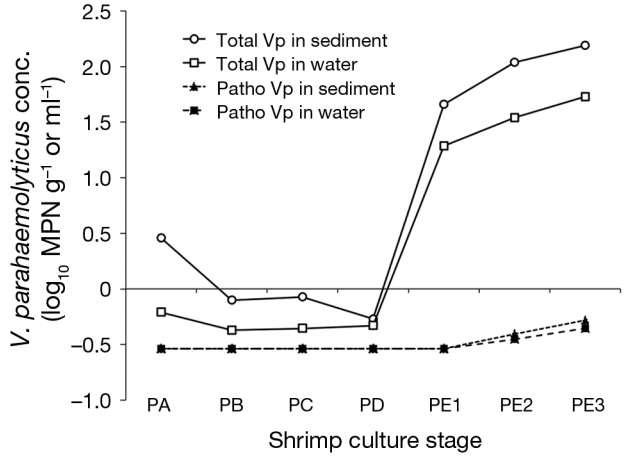

Fig. 2. Mean concentration of total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (patho Vp) in the pond sediment and water. PA: pond preparation; PB: water filling; PC: water treatment; PD: postlarva release; PE1: 1.5 mo culturing stage; PE2: 3 mo culturing stage; PE3: 4.5 mo culturing (harvest) stage. MPN: most probable number

detected in the natural feed and fresh feed, respectively (Table 2). Natural feed contained artemia and spirulina whereas fresh feed was composed of bloodworm, sandworm, squid, and shell. Thus, it is not surprising that *V. parahaemolyticus* was detected mostly in the fresh feed. The average concentrations of the total Vp detected were between −0.47 ± 0.40 and 1.45 ± 0.04 $\log_{10}$ MPN g^{-1} or ml^{-1} (Fig. 3). No *V. parahaemolyticus* was observed in the formulating feed (−0.53 ± 0 $\log_{10}$ MPN g^{-1}).

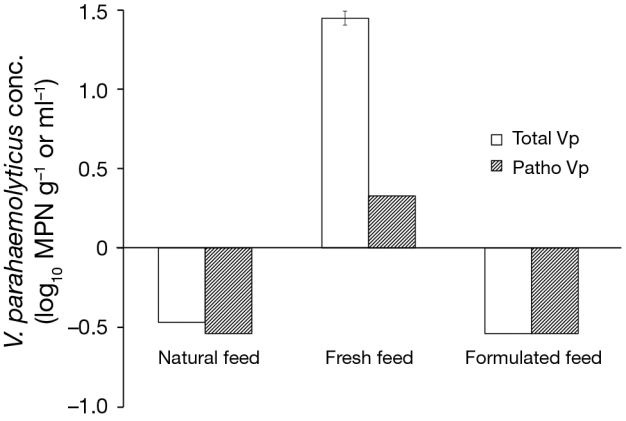

Fig. 3. Mean concentration of total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (pathoVp) in feed. Natural feed: artemia and spirulina; fresh feed: bloodworm, sandworm, squid, and shell. MPN: most probable number

Prevalence and concentration of *tdh*$^+$ Vp

Using the PCR technique for detection of pathogenic *V. parahaemolyticus* in broodstock, 5 samples and 1 sample were positive for *tdh*$^+$ Vp and *trh*$^+$ Vp, respectively, whereas using the LAMP technique, an additional *trh*$^+$ Vp was detected (Table 2). No pathogenic *V. parahaemolyticus* was detected in the egg samples or in any of the larval stages by either the PCR or the LAMP technique. At the PA–PD stages, all samples were negative for *tdh*$^+$ Vp and *trh*$^+$ Vp, but these were detected during the PE1–PE3 stages by the LAMP technique (Table 2). The prevalence of *tdh*$^+$ Vp increased from 1.2 to 11.4 and 15.9% for the PE1, PE2 and PE3 stages, respectively. Around 4.6% of *trh*$^+$ Vp was detected in the harvest stage. From the fresh feed, 26.7% of samples were positive for *tdh*$^+$ Vp as determined by the PCR technique whereas, using the LAMP technique, the number of positive samples increased to 53.3%. In addition, 20.0% of samples were found to be positive for *trh*$^+$ Vp using this technique.

In order to compare the sensitivity and specificity of PCR to the LAMP technique for detection of pathogenic *V. parahaemolyticus*, a total of 377 positive samples of *V. parahaemolyticus* were investigated (Table 2). PCR detected *tdh*$^+$ Vp and *trh*$^+$ Vp in 9 and 1 samples, respectively, whereas LAMP detected *tdh*$^+$ Vp and *trh*$^+$ Vp in 38 and 9 samples, respectively (Table 3). In addition, 339 and 368 samples were negative for the *tdh* and *trh* genes, respectively, by both LAMP and PCR. Thus, using PCR as the standard method for detection of virulent genes of *V. para-*

Table 3. Sensitivity and specificity of loop-mediated isothermal amplification (LAMP) compared to PCR for detection of pathogenic *Vibrio parahaemolyticus*

Test		*tdh*-PCR or *trh*-PCR Positive	Negative	Total	Sensitivity (%)	Specificity (%)
tdh-LAMP	Positive	9	29	38	100	92.1
	Negative	0	339	339		
	Total	9	368	377		
trh-LAMP	Positive	1	8	9	100	97.9
	Negative	0	368	368		
	Total	1	376	377		

haemolyticus, sensitivity and specificity of *tdh*-LAMP were 100 and 92.1%, respectively, whereas sensitivity and specificity of *trh*-LAMP were 100 and 97.9%, respectively (Table 3).

The average concentrations of pathogenic *V. parahaemolyticus* detected in broodstock, eggs, larva, PL, and shrimp were investigated. The highest concentration (0.16 ± 0.03 $\log_{10}$ MPN g^{-1}) was detected in shrimp at the PE3 stage (Fig. 1). In the pond cycle, the concentrations of pathogenic *V. parahaemolyticus* in the sediment and water increased simultaneously during the PE2 and PE3 stages (Fig. 2). In the feed, pathogenic *V. parahaemolyticus* was detected only in the fresh feed with an average concentration of 0.30 ± 0 $\log_{10}$ MPN g^{-1} (Fig. 3).

The probability of detection of total Vp or pathogenic *V. parahaemolyticus* in shrimp from at least one source of contamination fluctuated from the broodstock to the harvest stage (Fig. 4). This finding was compatible with the prevalence and concentration of *V. parahaemolyticus* detected in those stages (Table 2, Figs. 1 & 2). It appeared that the probability of detection of pathogenic *V. parahaemolyticus* was much lower than that of total Vp, particularly in the egg and nauplii stages, and to a lesser extent during the PL to PD stages. The correlation between total Vp and the pathogenic *V. parahaemolyticus* was observed from the protozoea to the PE3 stages, indicating the association between the concentrations of the total Vp and tdh^+ Vp and trh^+ Vp in those stages. However, according to the Pearson's correlation coefficient, a direct correlation between total Vp and pathogenic Vp was detected only during the PE3 stage (r = 0.76) (Fig. 5).

The uncertainty of probability was scrutinized by Monte Carlo simulation; it was found that the probability of detection of pathogenic *V. parahaemolyticus* was significantly lower than that of total Vp in the egg, nauplii, PA, PC, and PD stages ($p < 0.05$). Surprisingly, the probability of detection of pathogenic *V. parahaemolyticus* was statistically different from that of total Vp in the PE1 stage ($p < 0.05$) since the uncertainty of probability of detection of total Vp was not detected from the simulation. A sensitivity analysis of probability was performed using the correlation between probability and its input variables such as water, sediment, larva, etc. The correlations between

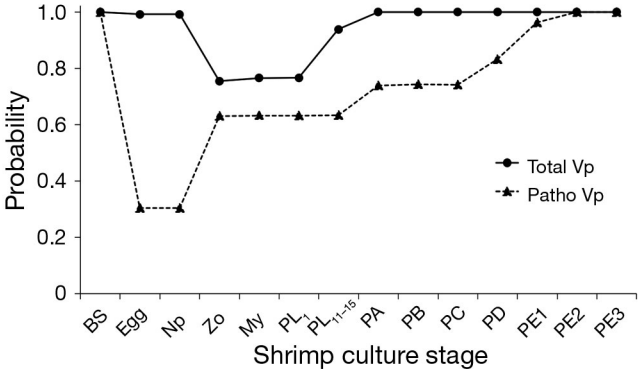

Fig. 4. Probability of detection of total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (patho Vp) in shrimp culture. BS: broodstock; Np: nauplii; Zo: protozoea; My: mysis; PA: pond preparation; PB: water filling; PC: water treatment; PD: postlarva release; PL_1: postlarva Day 1; $PL_{11–15}$: postlarva Day 11–15; PE1: 1.5 mo culturing stage; PE2: 3 mo culturing stage; PE3: 4.5 mo culturing (harvest) stage

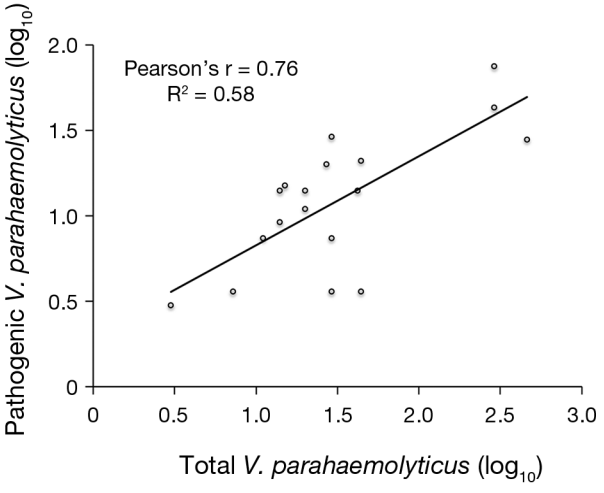

Fig. 5. Correlation between total *Vibrio parahaemolyticus* (total Vp) and pathogenic *V. parahaemolyticus* (patho Vp). Each dot represents 1 sample (n = 18); the straight line represents the best-fit line obtained by linear regression analysis

the probability of pathogenic *V. parahaemolyticus* and the prevalence of pathogenic *V. parahaemolyticus* in the water and sediment were between 0.60 and 0.67, particularly in the PA, PC, and PD stages (data not shown). The correlations between probability of pathogenic *V. parahaemolyticus* and prevalence of pathogenic *V. parahaemolyticus* in PL or shrimp and feed were around 0.37–0.40. Thus, we concluded that the important factors involved in the probability of detection of pathogenic *V. parahaemolyticus* in shrimp culture are water and sediment rather than PL or shrimp and feed.

DISCUSSION

In this study, the prevalence and concentration of the total and pathogenic *Vibrio parahaemolyticus* were determined during a full shrimp culture cycle using the MPN technique. In the hatchery cycle, it was clearly demonstrated that broodstock, eggs, and nauplii were the predominant sources of total Vp (Table 2, Fig. 1). Nonetheless, this bacterium may be a part of shrimp normal flora, and vertical transmission of organisms from broodstock to nauplii is common (Pangastuti et al. 2010). We found that around 83% of fresh feed that was provided to the broodstock for around 1 mo before spawning were positive for total Vp (Table 2). Thus, a repeat contamination of this organism at the broodstock stage might come from the fresh feed and could be transferred to the nauplii during larval rearing.

In this study, the prevalence and concentration of *V. parahaemolyticus* in the protozoea, mysis and PL stages was low because the hatching nauplii were filtered and transferred to a new tank (rearing tank) with new water supplied continuously. In addition, all the feed supplied in those stages were live or boiled artemia, spirulina, and formulated feed that for the most part had no *V. parahaemolyticus* (–0.53 $\log_{10}$ MPN g^{-1} or ml^{-1}) (Table 2, Fig. 3). Only one sample of artemia was positive for *V. parahaemolyticus*.

In the pond cycle during the PA–PC stages, management of the pond was quite good as less than 50% of the samples were positive for total Vp (Table 2). The sediment had more positives than the water samples (Fig. 2). However, during the PE1–PE3 stages, the prevalence and concentration of *V. parahaemolyticus* increased continuously. This may be due to repeat contamination of this bacterium from the PL released into the pond (PD stage) because 3 out of 24 samples of PL (12.5%) were positive for total Vp (data not shown). Together with the *V. parahaemolyticus* in the pond and the organic matter from the formulated feed supplied for shrimp in this culture cycle, these could be the important factors responsible for the increase of this bacterium during the PE1–PE3 stages. Most of the formulated feed was composed of ground and sieved fish, soybean, starch, fat, and oils.

In this study, the use of the LAMP technique for detection of pathogenic *V. parahaemolyticus* in the supernatant of the positive MPN tubes produced greater numbers than the colony PCR, as it detected an additional *trh*$^+$ Vp sample in the broodstock, detected *tdh*$^+$ Vp and *trh*$^+$ Vp in samples obtained during the PE1 to PE3 stages, and showed higher numbers of positive samples for *tdh*$^+$ Vp and detected *trh*$^+$ Vp in samples of fresh feed (Table 2). It is unlikely that this increase in detection is due to nonspecific detection because all of the positive tubes were confirmed for the presence of *V. parahaemolyticus*. In this work, in comparison to PCR, the sensitivity of LAMP for detection of both *tdh* and *trh* was 100% whereas the specificity for detection of *tdh* and *trh* were 92.1 and 97.9%, respectively (Table 3). These estimates are, however, dependent upon the assumption that PCR is a gold standard test, and the estimates may, therefore, be biased. The LAMP technique can also discriminate between the *trh* subgroups (Yamazaki et al. 2010). In this study, all 4 samples obtained from the PE3 samples were positive for *trh*2 whereas only 1 and 2 samples obtained from fresh feed were positive for *trh*1 and *trh*2, respectively (Table 2).

In our study, the probability of detection of total Vp and pathogenic *V. parahaemolyticus* were evaluated to assess their correlations. The probability of detection of pathogenic *V. parahaemolyticus* was lower than that of total Vp in the early period of the hatchery cycle (egg and nauplii stages) and the pond cycle (PA, PC, and PD stages). In addition, a direct correlation between total Vp and pathogenic *V. parahaemolyticus* was observed only during the PE3 stage (Fig. 5). Although these stages constituted less than half of the total stages in the shrimp culture, this indicated that total Vp might not be a good candidate to represent the pathogenic *V. parahaemolyticus* in the shrimp culture.

In this work, the factors involved in probability evaluation were the detection and enumeration of *V. parahaemolyticus* from various sources in each stage of shrimp culture. Therefore, identification of the sources contaminated with pathogenic *V. parahaemolyticus* either from the environment or from shrimp was of interest. We found that the correlation

between the probability of detection of pathogenic *V. parahaemolyticus* from the environment (water and sediment) was higher than that of detection from shrimp (r = 0.60–0.67). This indicated that the important sources of pathogenic *V. parahaemolyticus* were water and sediment.

After 4.5 mo of shrimp culture (PE3), the average concentrations of both the total Vp and the pathogenic Vp were higher than those detected after 1.5 and 3 mo culture (PE1 and PE2, respectively) (Fig. 2). During the PE3 stage, the maximum concentration of the total Vp was 4.0 ± 0.48 $\log_{10}$ MPN g^{-1} or ml^{-1}, and it was 3.1 ± 0 and 3.1 ± 0.48 $\log_{10}$ MPN g^{-1} or ml^{-1} in the PE1 and PE2, respectively. For the pathogenic Vp, the maximum concentration detected in PE3 was 1.2 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1}, and it was 0.5 ± 0 and 0.8 ± 0 $\log_{10}$ MPN g^{-1} or ml^{-1} in the PE1 and PE2, respectively. A correlation between the total Vp and pathogenic Vp was detected only in the PE3 stage (Fig. 5). Thus, the correlation in terms of the concentration between the total and pathogenic *V. parahaemolyticus* might be more apparent, particularly when the concentrations of the total and pathogenic *V. parahaemolyticus* reached a higher level of contamination.

Recently, *V. parahaemolyticus* has been demonstrated to be one of etiologic agents of acute hepatopancreatic necrosis disease (AHPND) (Tran et al. 2013). Outbreaks in shrimp culture occur within the first 30 d after releasing PL into the shrimp pond. Mass mortality can exceed 70% of the total shrimp and cause mass economic losses for the shrimp industry. However, *V. parahaemolyticus* that causes AHPND, lacks both the *tdh* and *trh* virulence genes, and it has not been reported to be associated with any human disease. The scenario used in this study will be useful for illustrating the source of this bacterium as well as of other shrimp pathogen contamination during the shrimp culture stages.

In conclusion, this study has illustrated that in shrimp culture, the prevalence and concentration of *V. parahaemolyticus* was high at the beginning of the hatchery cycle, and the presence of this organism decreased in the subsequent larval and PL stages. In contrast, they were low during the beginning of the pond cycle, yet dramatically increased in the later stages of culture. Broodstock and fresh feed were important sources of *V. parahaemolyticus*; however, water and sediment were involved in the probability of detection of pathogenic *V. parahaemolyticus.* A direct correlation between total and pathogenic *V. parahaemolyticus* was observed in the harvest stage. This study will be useful as a guideline to establish a level for the presence of pathogenic strains which is considered safe and, thus, decrease the risk for people who consume shrimp.

Acknowledgements. This work was supported by funds from the National Science and Technology Development Agency (NSTDA), Thailand (TG-CPMO 01-54-001) and the Thailand Research Fund through the Royal Golden Jubilee PhD Program (grant no. PHD/0066/2550). Thanks to Dr. Brian Hodgson for assistance with the manuscript.

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Appendix

For investigation of the probability of detection of *Vibrio parahaemolyticus* in samples, P_c represents the probability of the presence of a pathogen in a sample unit and P_s represents the prevalence of contaminated samples; thus, the probability of exposure to *V. parahaemolyticus* from a sample unit assay, P_e (Cassin et al. 1998), is:

$$P_e = P_c \times P_s \quad \text{Eq. (1)}$$

The probability of detecting *V. parahaemolyticus* in a sample depends on the concentration of this bacterium (C) in each sample type of each culture stage and the quantity of the corresponding sample tested (N; g^{-1} or ml^{-1} of sample). The concentration variable was described by lognormal distribution (μ, σ). This probability is assumed to follow a Poisson distribution (Crépet et al. 2007):

$$P_c = 1 - e^{(-NC)} \quad \text{Eq. (2)}$$

The prevalence (P_s) of contaminated sample depends on numbers of both contaminated samples (s) and sample size (k). In order to avoid problems that arise from 0% prevalence, the expected value from the beta distribution was used as the prevalence since the domain of the beta distribution encompasses all possible values of prevalence. The 2 parameters of the beta distribution are α ($\alpha = s + 1$) and β ($\beta = k - s + 1$) (Murray et al. 2004):

$$P_s = \text{Beta}(s + 1, k - s + 1) \quad \text{Eq. (3)}$$

From Eqs. (1) & (2), both P_c and P_s were the basis of contamination of *V. parahaemolyticus* in a single sampling. If repeated samplings (with an equal number of sample units in all sets of sampling) have been drawn k times, the probability of having *V. parahaemolyticus* at least 1 time from total k times was calculated as the following (Geng et al. 1983):

$$P_+ = 1 - (1 - P_e)^k \quad \text{Eq. (4)}$$

During the shrimp cultivation, *V. parahaemolyticus* could be derived from shrimp itself or from some other possible source in the environment, e.g. sediment, feed, water, etc. It would be interesting to determine the probability of detecting *V. parahaemolyticus* from at least 1 source in each stage of cultivation. With the same analogy of Eq. (4), each sampling equation time was regarded as each source of this bacterium. In this case, i would represent the number of possible sources of *V. parahaemolyticus* and P_{e_i} would represent P_e of each possible source of *V. parahaemolyticus*. Therefore, the probability of detecting *V. parahaemolyticus* in at least 1 source from total k sources of this bacterium in each stage of shrimp cultivation was slightly redefined as the following:

$$P_+ = 1 - \prod_{i=1}^{k} (1 - P_{e_i})^{k_i} \quad \text{Eq. (5)}$$

In this study, for a sample containing no *V. parahaemolyticus*, 0.29 MPN g^{-1} (or ml^{-1}) will be used for the calculation (FDA 2011). In the case where all samples are positive for *V. parahaemolyticus*, the average concentration of this bacterium was increased by 1 significant number. For example, if the upper limit of the MPN estimation was 1100 MPN g^{-1} (or ml^{-1}), then the average concentration of *V. parahaemolyticus* was 1200 MPN g^{-1} (or ml^{-1}) (FDA 2011).

The uncertainty of probability was described by the Monte Carlo simulation. The simulation was aimed at resampling all possible values of input variables such as concentration and prevalence by using the @Risk Decision tool version 4.5 (Palisade Corp.). The resamplings were repeated up to 10000 iterations.

Correlation between total Vp and tdh^+ Vp and trh^+ Vp was determined by Pearson's product-moment correlation.

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: November 21, 2013; Accepted: August 25, 2014
Proofs received from author(s): November 8, 2014